Rapid in vitro immobilisation of purified *Treponema pallidum* (Nichols strain), and protection by extraction fluids from rabbit testes

H J H Engelkens, M Kant, P C Onvlee, E Stolz, J J van der Sluis

Abstract

The use of Percoll-purified treponemes in an assay similar to the *Treponema pallidum* Immobilisation test demonstrated that immobilisation of purified treponemes by seronegative normal human serum proceeded at a much higher rate than that of unpurified treponemes. This suggests that the removal of the testicular extract makes the treponemes more vulnerable to this action. A preincubation of the purified treponemes with the testicular extract from infected or uninfected testes delayed their rate of immobilisation to that demonstrated by the unpurified treponemes. This showed that substances produced during the infection are probably not responsible for the delay in immobilisation. Discrimination between the classical and the alternative pathway of complement activation, studied by the ethylene glycol-bis (beta-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) method, showed that the classical pathway was responsible for the rapid immobilisation of the purified treponemes. However, the slow immobilisation in the EGTA-serum samples suggested a minor role of the alternative pathway in the immobilisation of the purified treponemes. Since the testicular extracts exerted an anti-complement activity, it needs to be investigated whether the protection offered to the purified treponemes by the testicular extracts is based on their deteriorating effect on the classical complement pathway or is due to a re-establishment of the protective cover around the treponemes.

The Department of Dermatology and Venereology, Erasmus University Rotterdam, and University Hospital Rotterdam-Dijkzigt, Rotterdam, The Netherlands
H J H Engelkens
M Kant
P C Onvlee
E Stolz
J J van der Sluis

Introduction

Syphilis caused by *Treponema pallidum* subspecies *pallidum* (*T pallidum*) can result in chronic disease. If left untreated, syphilis may result in disabling or even life-threatening situations during the tertiary stage, indicating that the host does not succeed in complete eradication of the treponemes. The mechanisms of the survival of treponemes in a host for many years are not yet fully understood. Besides a possible affection of the immune response,[1] there are several other hypotheses on how treponemes could possibly evade the host defence.

One hypothesis points to coverage of the treponeme with a protective layer, consisting of mucopolysaccharides and host proteins. The presence of mucopolysaccharides on treponemes in vitro was shown by Fitzgerald *et al*[2] and in rabbits by Zeigler *et al*.[3] Several serum proteins have been shown to be in close association with the outer membrane of *T pallidum*, isolated from infected rabbit testes.[4] However, in electron-microscopic studies the outer membrane presents itself as a symmetrical structure that shows no signs of extra-membrane components. Moreover, it was argued that the mere presence of substances on the outer surface of *T pallidum* does not prove that they play a role in providing protection to the organisms against the host defences.[5]

A more recent hypothesis focuses on the role of the structure of the outer membrane of *T pallidum* in the evasion of the treponemes from the immune response. It was shown by freeze fracture and deep etching techniques that the outer membrane contains only a small number of integral membrane proteins that can serve as targets for specific antibodies. These studies did not provide evidence that the surface of *T pallidum* is covered by an outer coat.[6]

As a consequence of both hypotheses it is assumed that treponemes can survive despite the sensitisation of the immune system, as demonstrated for instance by the presence of antitreponemal antibodies.

Treponemes for laboratory use are usually extracted from rabbit testes. As a consequence the treponeme suspensions are contaminated with rabbit components and possibly with substances produced during the infection. In 1984 it was shown by Hanff

et al[7] that density gradient centrifugation on Percoll gradients yielded suspensions of motile and virulent treponemes that were relatively free of host proteins.[7 8] The availability of this technique prompted us to compare the susceptibility to complement-dependent immobilisation of purified and unpurified treponemes, and to study the influence of the testicular extract on this susceptibility.

Material and methods

Propagation and extraction of T pallidum. Propagation and extraction of *T pallidum* was performed as previously described.[9] Briefly, the testes were minced and 1 ml of serum free basal reduced medium (BRM)[10] was added per gram of wet testicular tissue. The mixture was shaken for 45 minutes at room temperature in an atmosphere of 5% carbon dioxide and 95% nitrogen, and centrifuged for 10 minutes (800 × *g*) to sediment gross particulate matter. The fluid layer containing the treponemes was collected and part of it was used to prepare suspensions of "fresh" treponemes. The other part was centrifuged at 12000 × *g* at 4°C for 10 minutes to pellet the treponemes. The supernatant was carefully removed and saved. This centrifugation step reduced the number of treponemes in the supernatant to less than one per microscopic darkfield, a number that did not interfere with the final results of immobilisation experiments. The treponemes were resuspended in fresh BRM and subjected to Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation (43% Percoll in BRM) for 30 minutes at 37000 × *g* according to Hanff *et al.*[7] The layer containing the treponemes was collected and used to prepare suspensions of "Percoll" treponemes.

Enumeration of treponemes. The treponemes were counted using microslides (path length 0·05 mm, Camlab Limited, Cambridge, England, ref:5005) and the density of treponemes was calculated as previously described.[9]

Serum. One pool of human serum served as a complement source throughout this study. This pool was prepared from blood samples obtained from 50 donors all with a negative *T pallidum* Haemagglutination Assay (TPHA) test result. It was stored in small aliquots at −70°C. Samples used in the experiments were thawed only once. Serum from a patient with secondary syphilis (TPHA +, Venereal Disease Research Laboratory test 1:64, Fluorescent Treponemal Antibody-Absorbed test 3+) was used to isolate antitreponemal IgG (IgG(SII)), by DEAE-Sephadex-A 50 chromatography as described elsewhere.[11]

Modification of complement. Heat-inactivated serum was prepared by heating samples of the serum pool at 56°C for 30 minutes. Discrimination between the classical and the alternative pathway of complement activation was made by blockade of the classical pathway by the ethylene glycol-bis (beta-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) method for human sera. The efficacy of this procedure was demonstrated by the absence of lysis of optimally sensitised sheep red blood cells.[12] Proper functioning of the alternative pathway in the serum/EGTA buffer mixtures was verified by lysis of rabbit red blood cells as described by Platts-Mills and Ishizaka.[13]

Erythrocyte suspensions. Rabbit erythrocytes were washed three times in isotonic veronal buffer pH = 7·5 (VBS-buffer) and were used to prepare suspensions containing 1·5 × 10^8 erythrocytes per ml VBS buffer. Sheep erythrocytes suspended in Alsever's solution (Centraal Diergeneeskundig Instituut, Lelystad, The Netherlands) were washed three times in VBS-buffer containing 0·15 mM $CaCl_2$ and 1·0 mM $MgSO_4$ (VBS^{++}-buffer) and suspended at a density of 1 × 10^9 erythrocytes per ml. Optimal sensitisation was achieved with a 1:800 dilution of rabbit anti-sheep erythrocyte antibodies (Amboceptor, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands).

Immobilisation of treponemes. Suspensions of fresh treponemes were adjusted to a final density of 2 × 10^7 treponemes/ml, a final content of 10% v/v testicular extract and a final content of 25% v/v serum, by adding appropriate amounts of BRM and supernatant and serum pool. Mixtures containing 2 × 10^7 Percoll treponemes/ml and 5%, 10% and 20% v/v supernatant respectively were preincubated for 15 minutes. Subsequently 25% v/v serum was added. Aliquots of 0·5 ml of these mixtures were placed in small tubes, which were loosely plugged with cotton-wool and incubated in a reduced oxygen atmosphere at 34°C.[14] The percentage of mobile treponemes was determined in wet mounts after 0, 1, 2, 3·5, and 5·5 hours by observing at least 100 treponemes in randomly selected microscopic darkfields.

The immobilisation of Percoll treponemes by the classical and/or the alternative pathway of complement was studied as follows: mixtures of 25% v/v serum pool and 75% v/v VBS^{++} buffer and of 25% v/v serum pool and 75% v/v EGTA buffer were prepared. Similar mixtures were prepared using heat-inactivated serum samples. After 20 minutes at room temperature, an appropriate amount of Percoll treponemes was added to obtain a final density of 2 × 10^7 treponemes/ml. In control experiments it was verified that the Ca^{2+} ions present in the aliquot of Percoll treponemes did not abolish the blockade of the classical pathway by EGTA. Aliquots of these mixtures were stored and the percentage of mobile treponemes was determined after 0, 1, 2, 3, and 4 hours as described above.

Estimation of anti-complement capacity. The possible anti-complement activity of supernatants was inves-

tigated in a set-up analogous to the CH50 method for human sera.[12] Since BRM had been used in all immobilisation experiments, this buffer was used in these estimations. One ml of a 10-fold dilution of the serum pool caused approximately 50% haemolysis. This 10-fold dilution was used in the analysis of the effect of the supernatants on the complement-dependent haemolysis: 100 μl serum was mixed with 80 μl of the various supernatants and adjusted to 1·0 ml. This resulted in a serum:supernatant ratio of 5:4, representing the ratio that was used in the immobilisation experiments with the largest amount of supernatant. The haemolysis in these tubes was expressed as a percentage of the haemolysis obtained in the tubes with the 10-fold diluted serum.

Immunofluorescence. Immunofluorescence studies were performed on Percoll treponemes, which stick spontaneously to clean glass surfaces. The integrity of the outer membrane could be preserved by the addition of 0·025% v/v fetal calf serum (FCS) to the suspensions. Slides were prepared as follows: suspensions of Percoll treponemes were adjusted to 2×10^6 treponemes/ml and 0·025% (v/v) FCS was added. One ml was placed in a 35 mm plastic Petri dish (Costar) equipped with a clean coverglass. After centrifugation at 800 × *g* for 10 min, the coverglasses were rinsed in BRM and were overlaid with two drops of IgG(SII) and incubated at room temperature for 30 min. After rinsing the coverglasses were overlaid with two drops of goat anti-human IgG, working dilution 1:50, labelled with fluorescein isothiocyanate (FITC, Nordic, Tilburg, The Netherlands) and incubated for 30 min at room temperature. Coverglasses were rinsed again and then placed upside down on microscopic slides and sealed with nail polish. They were read immediately. This procedure allowed for the adherence of approximately 70% of treponemes originally present. Absence of fluorescence with the monoclonal antibody (MoAb) CC 9,[15] which is directed against the axial filaments of the treponemes, was used as a control on the integrity of the outer membrane.[16] Microscopic equipment was as described previously.[11]

SDS-PAGE and Western Blotting. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting were performed using a mini-apparatus (Biorad, Richmond, California) according to the instructions of the manufacturer. Stacking and separating gels consisted of 5% and 12% acrylamide respectively. Approximately 4×10^7 Percoll-purified treponemes were suspended in sample buffer composed of 0·0625 M Tris-HCl (pH 6·8), containing 2% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and bromophenol blue as a tracking dye, and were heated in a boiling water bath for 4 min. These were electrophoresed until the dye front reached the bottom of the gel and the polypeptides were transferred to Immobilon PVDF membranes (Millipore, Bedford, Massachusetts) during 1 h. The membrane was cut into 20 strips, resulting in approximately 2×10^6 solubilised treponemes per strip. Strips were incubated for 2 hours in a 200-fold dilution of the serum pool and a 50-fold dilution of the supernatants respectively to detect IgG anti-treponemal antibodies in these fluids. Anti-treponemal antibodies associated with the treponemes were detected with affinity-purified goat anti-rabbit IgG (Southern Biotechnology Associates, Birmingham, Alabama). Immunochemical staining was visualised by incubation for 2 h with the appropriate gold-labelled conjugates, followed by silver enhancement. Direct staining of the polypeptides was performed with Aurodye Forte. Blocking of non-specific protein-binding sites of the membranes, preparation of primary antibody and conjugate dilutions and of the washing solutions were done as recommended by the manufacturer (Janssen Life Sciences Products, Beerse, Belgium). Non-reactivity of the conjugates with the treponemal polypeptides was controlled by incubation in PBS/Tween 20 (0·05%) instead of primary antibody, followed by the appropriate conjugate. The low-molecular weight standards from Sigma (St Louis, USA) were used in estimating the size of the treponemal polypeptides.

Statistical analysis. In the evaluation of results, Spearman's correlation coefficient was used.

Results

Investigations into the complement-dependent immobilisation of the fresh treponemes using human serum showed a gradual time-dependent decline in the mobility of the treponemes (fig 1). After 5·5 h of incubation, approximately 40% of the treponemes was still mobile. On the other hand, the Percoll treponemes had lost their mobility almost completely after 2 h of incubation. The controls with heat inactivated serum showed a good mobility of the treponemes: the mobility of the fresh and Percoll purified treponemes was more than 96% after incubation for 5·5 h (not shown). This demonstrates the complement-dependent nature of the immobilisation.

Pre-incubations of the Percoll treponemes with autologous supernatants of rabbit testes caused a rescue in their complement-dependent immobilisation. As shown in fig 1, immobilisation of the treponemes in the supernatant-containing samples progressed at a similar rate as that of the fresh treponemes. After the incubation period of 5·5 h the mean percentages of mobile treponemes were 24%, 30% and 32% in the samples containing 5%, 10% and 20% supernatant respectively. This indicates a slight dose-dependent influence of the supernatants.

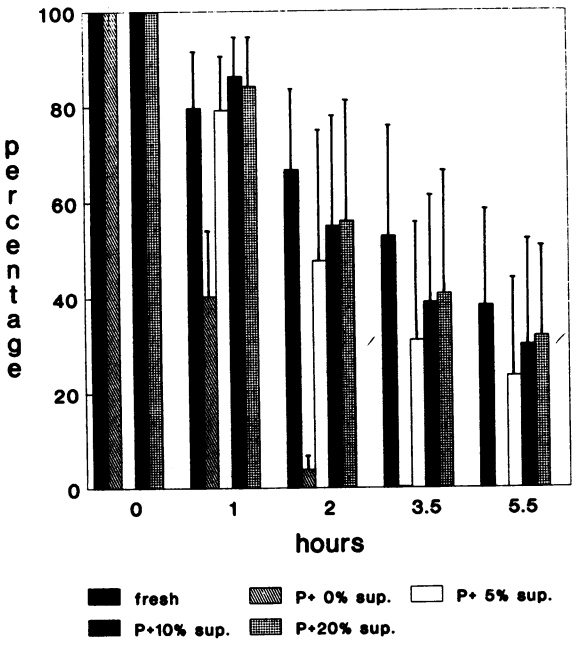

Figure 1 Complement-dependent immobilisation of fresh and Percoll-purified (P + 0%) treponemes and the effect of 5% (P + 5%), 10% (P + 10%) and 20% (P + 20%) supernatant from infected testes on the immobilisation of the Percoll-purified treponemes. Human serum was used as a source of complement. Results are expressed in percentage mobile treponemes. Means and standard deviations of six treponeme suspensions isolated from different rabbits are shown.

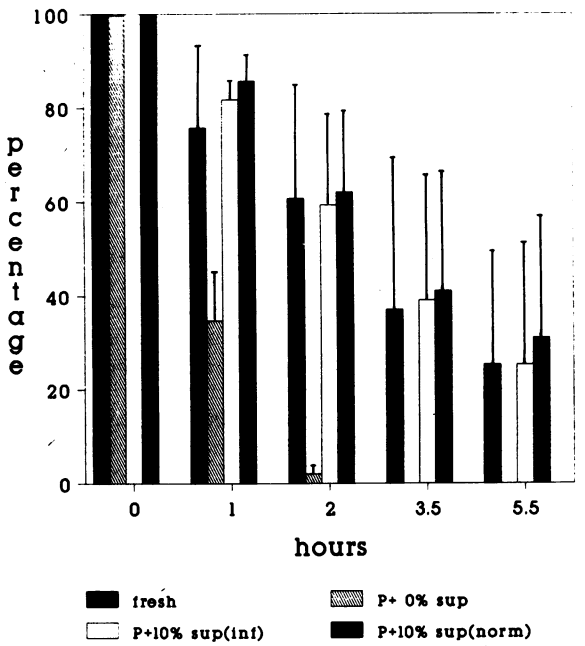

Figure 2 Complement-dependent immobilisation of Percoll-purified treponeme suspensions containing 10% supernatant from infected (P + 10% sup (inf)) or uninfected (P + 10% sup (norm)) rabbit testes and Percoll-purified (P + 0% sup) and fresh treponemes. Results are expressed in percentage mobile treponemes. Means and standard deviations of three treponeme suspensions isolated from different rabbits are shown.

Again, the treponemes incubated with heat-inactivated serum had a mobility of at least 96% in all supernatant-containing mixtures after 5·5 h of incubation.

Experiments in which the preincubations were performed with supernatants prepared from uninfected rabbit testes (fig 2) demonstrated that these supernatants interfered similarly with the immobilisation of the Percoll treponemes as did the autologous supernatants from infected rabbit testes. The Percoll treponemes pre-incubated with supernatants from uninfected rabbit testes showed at all times a slightly higher percentage of mobile treponemes than Percoll treponemes pre-incubated with supernatants from infected testes.

The results after blocking the classical complement pathway are shown in fig 3. When untreated serum was used as complement source the Percoll treponemes were rapidly immobilised. However, when EGTA-serum was used the immobilisation of the Percoll treponemes progressed at a much slower rate. When heat-inactivated serum or heat-inactivated EGTA-serum was used, the Percoll treponemes survived well during the observation period of 4 h. This demonstrates that removal of Ca^{2+}-ions was not responsible for the immobilisation of the treponemes during the observation period. Since an effective blockade of the classical pathway and a proper functioning of the alternative pathway in EGTA-serum was verified, the absence of rapid immobilisation of the Percoll treponemes demonstrates that the classical pathway is the major route by which the treponemes are immobilised. However, the slow immobilisation in EGTA-serum samples indicates that the alternative pathway can also contribute to the immobilisation of the Percoll treponemes.

It was realised that the delay in immobilisation caused by the supernatants could be based on anti-complement action of the latter. The addition of supernatants used in fig 1 to the complement source lowered its haemolytic capacity to 77·8, SD 4·4% of its capacity without supernatant. With the supernatants from uninfected testes the result was 84·6, SD 6·0%. However, the expectation that a less effective complement cascade would result in less rapid immobilisation of the Percoll treponemes could not be proven by statistical analysis: no correlation was found between these two parameters at any of the times indicated in fig 1.

In immunofluorescence investigations with MoAb

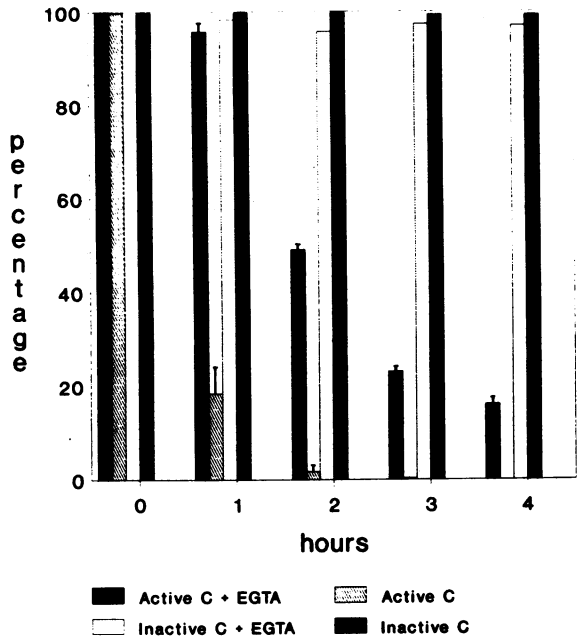

Figure 3 Complement-dependent immobilisation of Percoll-purified treponemes in untreated serum (active C), serum treated with EGTA to block the classical pathway (active C + EGTA) and the heat-inactivated counterparts (inactive C and inactive C + EGTA respectively). Results are expressed in percentage mobile treponemes. Means and standard deviations of three suspensions isolated from different rabbits are shown.

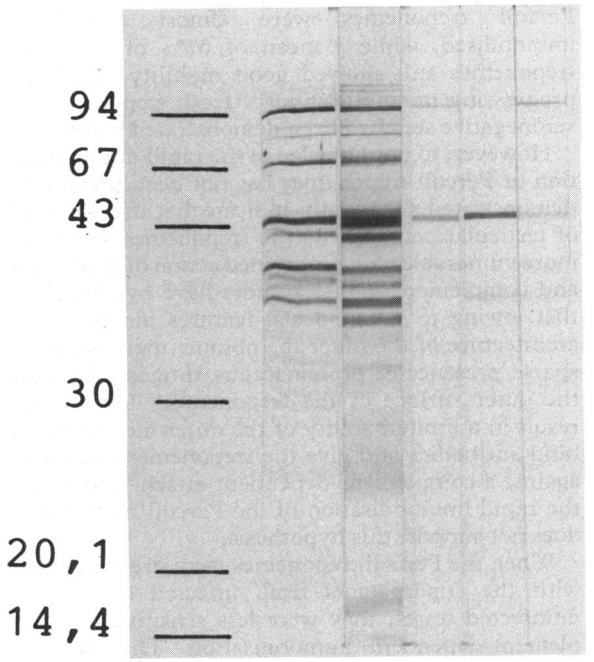

Figure 4 Western immunoblot analysis of antibodies against treponemal antigens in the serumpool and testicular supernatants. Solubilised treponemes purified by Percoll density centrifugation were used. From left to right: lanes 1–5. Lane 1: Treponemal polypeptides stained with Aurodye to visualise all polypeptides. Lane 2: Treponemal polypeptides stained with IgG (SII), followed by gold-labelled goat anti-human IgG and silver enhancement. Lane 3: Treponemal polypeptides stained with 1:200 diluted human serum pool, and stained with gold-labelled goat anti-human IgG, followed by silver enhancement. Lanes 4 and 5: Two representative examples of treponemal polypeptides incubated with 1:50 diluted supernatants from infected rabbit testes and stained with gold-labelled goat anti-rabbit IgG, followed by silver enhancement. The location of the molecular mass markers is indicated on the left.

CC 9, no fluorescence was observed. This points to an intact outer membrane. With the polyclonal IgG (SII) preparation, over 95% of the Percoll treponemes showed a weak positive fluorescence, often of a speckled character. This demonstrates the accessibility of the outer membrane of the Percoll treponemes to antibodies.

The presence of IgG class antibodies in the supernatants, in the human serum pool and on the Percoll-purified treponemes was investigated by means of SDS-PAGE and Western Blotting (fig 4). Antitreponemal IgG antibodies were readily detected in the serum pool and the supernatants, of which two examples are shown (lanes 4,5). IgG antibodies strongly reactive to 44 kD treponemal polypeptides were common in these fluids. In addition, IgG antibodies to 94, 78, 67, and 39 kD polypeptides were detected in the human serum pool. The supernatants contained IgG antibodies to 94, 39 and 36 kD polypeptides. Detection of antibodies on the Percoll treponemes was less clearcut. Only occasionally very faint indications of anti-IgG conjugate-stained polypeptides of molecular mass of 50 kD, corresponding to the heavy chain of IgG, were observed (not shown).

Discussion

A large part of the hypothesis that *Treponema pallidum* is covered by a protective extra-cellular layer has been deduced from serological reactions in which live treponemes are used, for example the *Treponema Pallidum* Immobilisation (TPI) reaction. During the long incubation times needed to immobilise the treponemes, the microorganisms are supposed to lose their protective cover, making them susceptible to the combined action of anti-treponemal antibodies and complement. The availability of a technique to purify the treponemal suspensions from the rabbit components offered the opportunity to compare the periods of time needed for the immobilisation of purified and unpurified microorganisms. The purification of the treponemes drastically changed their behaviour towards complement-dependent immobilisation: after 2 h the

Percoll treponemes were almost completely immobilised, while a mean of 67% of the fresh treponemes still showed good mobility. A slowly progressing immobilisation of fresh treponemes in seronegative sera had been demonstrated before.[17]

However, to our knowledge the rapid immobilisation of Percoll treponemes has not been previously demonstrated. Our results indicate that after removal of testicular components the treponemes are much more vulnerable to the combined action of antibodies and complement. Some authors have hypothesised that, owing to some special features innate to the architecture of the outer membrane, there is only a sparse presence of proteinaceous antigenic sites on the outer surface of the treponemes. This would result in a limited ability of the outer membrane to bind antibodies and give the treponemes resistance against a complement-dependent attack.[6] However, the rapid immobilisation of the Percoll treponemes does not support this hypothesis.

When the Percoll treponemes were pre-incubated with the supernatants from infected as well as uninfected testes, they were less sensitive to complement-dependent immobilisation. This demonstrates: firstly that a delay in immobilisation caused by supernatants is possible, and secondly that this delay is not dependent on the presence of infection in the testes from which the supernatant was derived. This rules out a role of components present in the testicular tissue, produced as a result of the treponemal infection. Previously, it was demonstrated that a treponemal infection in the rabbit testes is accompanied by the production of a large amount of hyaluronic acid, which is easily extracted from the minced testicular tissue.[9] This compound, together with other acid mucopolysaccharides is often regarded as a substance which could possibly provide protection to the treponemes.[2-3 9 18-20] The present experiments make a major role of hyaluronic acid in the delay of complement-dependent immobilisation unlikely.

This delay caused by the supernatants could be accomplished in several ways:

Firstly, the supernatants could have an anti-complement action and, particularly relevant to our studies, lower the effectivity of the classical pathway of the activation of complement. A limited reduction in the capacity of the serum pool to haemolyse optimally sensitised sheep erythrocytes in the presence of supernatants was demonstrated. This shows an anti-complement capacity of the supernatants used. However, it is doubtful whether this reduction in complement level can explain the delay in immobilisation caused by the supernatants. It would be expected that, the more markedly the complement level is affected, the larger the delay in immobilisation of the treponemes should be. However, there was no correlation between the data to support this hypothesis. Therefore, a definite conclusion has to await experiments which demonstrate that it is possible to separate the component(s) delaying the immobilisation of the treponemes from those affecting the complement level. A second possibility is that treponemal antigens present in the supernatants obtained from infected rabbit testes can occupy binding places of antibodies present, thereby inhibiting the binding to the treponemes, thus influencing immobilisation characteristics. However, this possibility seems unlikely because in experiments in which supernatants from uninfected rabbit testes were used a similar level of delay in immobilisation was observed. A third possibility is that the delay in immobilisation of the purified treponemes is accomplished by the occupation of relevant antigenic sites on the treponemal outer membrane by components present in the supernatants. This would prevent formation of antigen-antibody complexes on the treponemal surface and in turn prevent the initiation of the classical complement cascade. The accessibility of the outer membrane of the Percoll treponemes to the anti-treponemal IgG (SII) antibodies was demonstrated by immunofluorescence studies. This is in contrast with fresh treponemes: earlier findings on the accessibility of the outer membrane of the fresh treponemes adhering to cultured fibroblast or layers of fibronectin, showed a fluorescence with a similar IgG preparation of only a limited percentage.[16] This difference in access of antibodies is compatible with the rapid, complement-dependent immobilisation of the Percoll treponemes as compared to that of the fresh treponemes.

As was discussed before by Fitzgerald, it is difficult to indicate which antibodies take part in the in vitro complement-treponeme interactions.[18] Freshly harvested, Percoll-purified treponemes still carry rabbit IgG class antibodies on their surface.[8] These antibodies might already have been present before the treponemes were harvested or have been collected during the extraction procedure. Our demonstration of anti-treponemal IgG class antibodies in Western blots of solubilised Percoll treponemes was doubtful. However, among the IgG class antibodies common to the supernatants and the human serum pool, there were antibodies directed to the treponemal polypeptides contained in the 44 kD region. Polypeptides of this size have been implicated as the surface antigens involved in the immobilisation of the treponemes. Murine MoAbs against 44 kD polypeptides have been shown to be capable of immobilisation.[21] The participation of the antibodies to the remaining polypeptides in this respect remains to be established.

In conclusion, it has been demonstrated that Percoll treponemes are rapidly immobilised by activation of the classical complement pathway.

Delay in this immobilisation can be provided by rabbit testicular components present in the extraction fluids. Further experiments will have to elucidate whether this delay is based on an anti-complement capacity of these extraction fluids or whether components in these fluids combine with the outer membrane of the purified treponemes in reconstructing a protective extra-cellular layer. In the latter case the present experimental design will permit an analysis of the substances that participate in the formation of this layer.

The authors are grateful to the personnel of the Laboratory Animal Centre, Erasmus University Rotterdam, for kind help in work with the rabbits, and to A Cockayne, University of Birmingham, United Kingdom, for CC9. We would like to thank Dr FCHA Kothe, Director of the Blood Bank, Rotterdam, The Netherlands, for providing donor sera.

Address for correspondence: HJH Engelkens, MD, Department of Dermatology and Venereology, Erasmus University Rotterdam, PO Box 1738, 3000 DR Rotterdam, The Netherlands.

1 Fitzgerald TJ. Pathogenesis and immunology of *Treponema pallidum*. *Ann Rev Microbiol* 1981;35:29–54.
2 Fitzgerald TJ, Johnson RC. Surface mucopolysaccharides of *Treponema pallidum*. *Infect Immun* 1979;24:244–51.
3 Zeigler JA, Jones AM, Jones RH, Kubica KM. Demonstration of extracellular material at the surface of pathogenic *T pallidum* cells. *Br J Venereal Dis* 1976;52:1–8.
4 Alderete JF, Baseman JB. Surface-associated host proteins on virulent *Treponema pallidum*. *Infect Immun* 1979;26:1048–56.
5 Hovind-Hougen K. Morphology. In: Schell RF, Musher DM, eds. *Pathogenesis and Immunology of Treponemal Infection*. New York: Marcel Dekker, Inc, 1983:3–28.
6 Radolf JD, Norgard MV, Schulz WW. Outer membrane ultrastructure explains the limited antigenicity of virulent *Treponema pallidum*. *Proc Natl Acad Sci USA* 1989;86: 2051–5.
7 Hanff PA, Norris SJ, Lovett MA, Miller JN. Purification of *Treponema pallidum*, Nichols strain, by Percoll density gradient centrifugation. *Sex Transm Dis* 1984;11:275–86.
8 Baughn RE. Antibody-independent interactions of fibronectin, Clq, and human neutrophils with *Treponema pallidum*. *Infect Immun* 1986;54:456–64.
9 Van der Sluis JJ, Van Dijk G, Boer M, Stolz E, van Joost Th. Mucopolysaccharides in suspensions of *Treponema pallidum* extracted from infected rabbit testes. *Genitourin Med* 1985;61:7–12.
10 Fieldsteel AH, Cox DL, Moeckli RA. Cultivation of virulent *Treponema pallidum* in tissue culture. *Infect Immun* 1981;32:908–15.
11 Van der Sluis JJ, van Reede EC, Boer M. Immunoglobulin G subclasses of fluorescent anti-treponema antibodies: evidence for a sequential development of specific anti-treponema IgG responses in patients with early syphilis. *J Clin Microbiol* 1986;24:418–23.
12 Austen KF, Bond HE, Chase MW, *et al*. Chapter 17. Complement. In: Williams CA, Chase MW, eds. *Methods in Immunology and Immunochemistry. Agglutination, Complement, Neutralization and Inhibition*. Vol 4. New York: Academic Press, 1977:127–274.
13 Platts-Mills TAE, Ishizaka K. Activation of the alternate pathway of human complement by rabbit cells. *J Immunol* 1974;113:348–58.
14 Van der Sluis JJ, Koehorst JAM, Boer AM. Factors that inhibit the adherence of *Treponema pallidum* (Nichols strain) to a human fibroblastic cell line: development in serum of patients with syphilis. *Genitourin Med* 1987;63:71–6.
15 Bailey MJ, Cockayne A, Penn CW. Production of murine monoclonal antibodies to the major axial filament polypeptide of *Treponema pallidum*. *J General Microbiol* 1987;133: 1805–13.
16 Van der Sluis JJ, Kant M, Onvlee PC, Stolz E. The inaccessibility of the outer membrane of adherent *Treponema pallidum* (Nichols strain) to anti-treponemal antibodies, a possible role of serum proteins. *Genitourin Med* 1990;66: 165–70.
17 Blanco DR, Radolf JD, Lovett MA, Miller JN. Correlation of treponemicidal activity in normal human serum with the presence of IgG antibody directed against polypeptides of *Treponema phagedenis* biotype Reiter and *Treponema pallidum*, Nichols strain. *J Immunol* 1986;137:2031–6.
18 Fitzgerald TJ. Activation of the classical and alternative pathways of complement by *Treponema pallidum* subsp. *pallidum* and *Treponema vincentii*. *Infect Immun* 1987;55:2066–73.
19 Christiansen S. Hypothesis. Protective layer covering pathogenic treponemata. *Lancet* 1963;1:423–5.
20 Alderete JF, Baseman JB. Serum lipoprotein binding by *Treponema pallidum*: possible role for proteoglycans. *Genitourin Med* 1989;65:177–82.
21 Bailey MJ, Cockayne A, Penn CW. Monoclonal antibodies directed against surface-associated polypeptides of *Treponema pallidum* define a biologically active antigen. *J General Microbiol* 1987;133:1793–803.